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Modulation of microglia polarization dynamics during diabetic retinopathy in *db*/*db* mice

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ABSTRACT

Retinal diseases linked to inflammation are often accompanied by macrophage/microglial cells activation. However, the dynamics between M1 (pro-inflammatory) and M2 (anti-inflammatory) polarization of microglia during diabetic retinopathy (DR) has not been investigated and it might be therapeutically useful. We assessed microglia polarization in retinas from *db*/*db* mice and human diabetic donors and also the microglia-mediated anti-inflammatory effects of the bicyclic nojirimycin derivative (1*R*)-1-dodecylsulfinyl-5*N*,6*O*-oxomethylidenenojirimycin (*R*-DS-ONJ). Visual function in mice was evaluated by electroretinogram (ERG). Expression of pro- and anti-inflammatory markers in the retina was analyzed by immunofluorescence, Western-blot and quantitative real-time PCR. Lipopolysaccharide (LPS)-mediated polarization profile was studied in Bv.2 microglial cells in the absence or presence of anti-inflammatory cytokines (IL4/IL13) or *R*-DS-ONJ. At 5 weeks of age, reduced ERG amplitude values of rod and mixed waves were detected in *db*/*db* compared to *db*/+ mice that correlated with elevated circulating endotoxemia and pro-inflammatory cytokines. At this early stage of DR, the marker of activated microglia Iba-1 co-localized with the M2 marker arginase-1 in the retina. Conversely, in retinas from 8 weeks old *db*/*db* mice Iba-1-colocalized with active caspase-1, a key component of the inflammasome, reflecting an opposite pattern of microglia polarization. Markers of activated microglia were detected in retinas of diabetic donors. Treatment of Bv.2 cells with LPS and IL4/IL13 or *R*-DS-ONJ switched the M1 response towards M2. In retinal explants from *db*/*db* mice, *R*-DS-ONJ induced a M2 response. In conclusion, the modulation of microglia polarization dynamics towards a M2 status at early stages of DR offers novel therapeutic interventions.

1. Introduction

Insulin resistance and chronic inflammation are implicated in the pathogenesis of type 2 diabetes (T2D) and in diabetic retinopathy (DR), its most prevalent complication [1]. In fact, neuroinflammation induced by the diabetic milieu is a central contributing factor in DR progression [2]. Similarly to other neurodegenerative diseases, DR exhibits characteristics of low-grade chronic inflammation [3] in which changes in retinal expression of inflammatory mediators occur in concert with functional changes in retinal permeability and apoptosis [4,5].

It has been proposed that in neuroinflammation microglia becomes activated and produce inflammatory mediators. Microglia, serving as resident macrophages of the retina, has multiple functional states and carries out diverse functions. Capable of rapid dynamism and motility,

Abbreviations: CNS, central nervous system; DR, diabetic retinopathy; ERG, electroretinogram; FELASA, Federation of European Laboratory Animal Science Associations; GCL, ganglion cell layer; GFAP, glial fibrillar acidic protein; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; INL, inner nuclear layer; JNK, *c*-Jun N-terminal kinase; LPS, lipopolysaccharide; mRNA, messenger ribonucleic acid; OP, oscillatory potentials; OPL, outer plexiform layer; OS, outer segment; p38 MAPK, p38 mitogen-activated protein kinase; qRT-PCR, quantitative Real-Time PCR; RNA, ribonucleic acid; RPE, retinal pigment epithelium; SEM, standard error of the mean; sp^2-iminosugar dodecylsulfoxide derivative *R*-DS-ONJ, C5; T2D, Type 2 Diabetes.

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microglial cells synthesize and release cytokines, chemokines, neurotrophic factors, and neurotransmitters that interact with multiple cell types in the central nervous system (CNS) and exert cytotoxic, cytoprotective and scavenger effects depending on the tissue context [6]. Retinal diseases such as proliferative DR and diabetic macular edema are often accompanied by macrophage/microglia cells activation [7,8].

The existence of a continuum of polarization states in macrophages results from the integration of the intracellular signals triggered by their surrounding milieu [9,10]. M1, or classically-activated macrophages, mainly secrete pro-inflammatory cytokines such as TNFα, IL12, IL23, IL1β, IL6 and chemotactic factors and are involved in the pro-inflammatory response. By contrast, alternatively-activated macrophages (M2) express high levels of arginase-1 and IL10, but low levels of IL12 and IL23 and are usually induced by the anti-inflammatory cytokines IL4 and IL13 [11].

DR exhibits many features of chronic inflammation such as increased NO production and release of pro-inflammatory cytokines [12]. In fact, TNFα, IL1β, IL-8 and MCP-1 have been found elevated in the vitreous of diabetic patients [13,14]. Recently, the inflammasome complex, which cleaves pro-IL1β into secreted IL1β via caspase-1, has been found activated in retinal pigment epithelial cells cultured under high glucose levels [15], but its modulation in the retina during DR has not yet been explored. In this scenario, microglia may underline its different functional properties and, therefore, targeting its polarization is a promising approach for the treatment of CNS diseases [16] including DR.

Among therapies targeting pro-inflammatory mediators, the natural compounds have emerged as an alternative to chemical drugs. Iminosugar glycosyl hydrolase inhibitors such as 1-deoxynojirimycin and castanospermine have a strong potential in therapies for cancer, viral infections, diabetes and glycosphingolipid storage disorders [17,18]. In particular, sp^2-iminosugar-type bicyclic nojirimycin analogues with an alpha-configured *N*-, *S*-, or *C*-linked pseudoanomeric group have been previously synthetized and evaluated as antitumor agents [18]. However, their effects in inflammatory processes during DR remain to be elucidated.

In recent years the C57BL/KsJ-*db*/*db* mouse model has been extensively used to investigate the pathogenesis of DR [19] since reproduce the neurodegenerative process that occur in the human diabetic retina [20]. However, the events that occur in the retina at early stages of DR previous to neurodegeneration, and, in particular the role of microglia associated to neuroinflammation in *db*/*db* mice remain unknown. The aim of this study is to examine the polarization of microglia during the early stages of DR and its modulation by a sp^2-iminosugar-type bicyclic nojirimycin analogue.

2. Methods

2.1. Reagents and drugs

Fetal bovine serum (FBS) and culture media were obtained from Invitrogen (Grand Island, NY, USA). Bovine serum albumin (BSA) and bacterial lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St Louis, MO, USA). IL4 and IL13 were purchased from Preprotech (London, UK). Bradford reagent, acrylamide, immunoblot PVDF membranes and chemiluminescent HRP Substrate were purchased from Bio-Rad (Madrid, Spain).

Antibodies against caspase-1 (p10) (sc-514), iNOS (sc- 650), IκBα (sc-371), JNK (sc-571), phospho-38 MAPK (Thr180/Tyr182) (sc-17,852-R) and p38 MAPK (sc-9212) were purchased from Santa Cruz Biotechnology (Palo Alto, CA, USA). Anti-phospho-JNK (Thr183/Tyr185) (#4668) antibody was purchased from Cell Signaling Technology (MA, USA). Anti-Iba-1 (ab5076) and anti-Cd11-b (ab8878) antibodies were from Abcam (Cambridge, UK). Anti-Arginase-1(BD 610708) was purchased from BD Biosciences (Madrid, Spain). Anti-GFAP antibody (z0334) was obtained from DAKO (Glostrup, Denmark) and anti-α-tubulin (T-5168) antibody was from Sigma Chemical Co. (St Louis, MO, USA).

2.2. Animals and retina isolation

db/*db* and *db*/+ male mice both in the C57BL/KsJ genetic background were purchased from Harlan (Harlan Laboratories, Inc. UK). Mice were maintained in light/dark (12-hours light/12-hours dark)-, temperature (22 °C)- and humidity-controlled rooms, and fed ad libitum with free access to water. All animal experimentation followed recommendations of the Federation of European Laboratory Animal Science Associations (FELASA) on health monitoring in accordance with the regulations of the Association for Research in Vision and Ophthalmology. Animals were killed by cervical dislocation and eyes were enucleated. The lens, anterior segment, vitreous body, retinal pigment epithelium and sclera were removed and the retinas were immediately frozen for protein or RNA extraction. The animals were sacrificed at 5, 6, 8, 12 or 20 weeks of age according to the experimental approach.

2.3. Retinal explants

Ex vivo assays were performed with retinas from 8 weeks old male *db*/+ and *db*/*db* mice as previously described [21]. Following isolation, retinas were cultured in R16 medium (provided by Dr. P.A. Ekstrom, Lund University, Sweden) with no additional serum. Retinas were stimulated with *R*-DS-ONJ (compound C5) at 50 μM for 24 h as indicated in the figure legends.

2.4. Human retinas

Retinas were obtained from the Tissue Bank of the Research Institute Vall d'Hebron (Barcelona, Spain). Eight type 2 diabetic and 8 non-diabetic donors matched by age and gender were included in the study. Clinical characteristics of donors have been previously detailed [22]. One eye-cup was harvested in order to separate neuroretina from retinal pigment epithelium and both tissues were immediately frozen in liquid nitrogen and stored at −80 °C. The procedure for eye-cup donation and for handling of this biologic material, approved by the ethical committee, was regulated by the protocol of donations of the Tissue Bank.

2.5. Endotoxin detection

Serum endotoxin concentrations were measured with a commercial kit (QAYEE-BIO; Deltaclon, Madrid, Spain) according to the manufacturer's instructions. Briefly, blood samples were collected in non-pyrogenic and endotoxin-free tubes, centrifuged at 2500 ×*g* for 10 min and serum was separated. Samples were diluted (1/5) with endotoxin-free water and horseradish peroxidase (HRP) was added. Samples were gently shacked, incubated for 60 min at 37 °C and then washed 5 times. Chromogen solution A was added to the samples that were gently shacked and further incubated for 10 min at 37 °C protected from light. Finally, stop solution was added and samples were measured at 450 nm.

2.6. Cell culture

Mouse microglia Bv-2 cell line was provided by Dr. M.L. Nieto (CSIC, Spain). Bv-2 cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in RPMI supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin (Sigma) and 2 mM L-glutamine (Gibco, Carlsbad, California, USA). Cells were grown up to 70% confluence, washed with PBS and further stimulated in serum-free medium with LPS (200 ng/ml) with or without a mixture of IL4/IL13 (20 ng/ml each; M2) or *R*-DS-ONJ (compound C5) for several time-periods.